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Determination of Human Granulocyte Elastase by the Immunoactivation Method on the Hitachi® 717 Automated Analyser

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Summary: This paper describes a fully mechanized homogeneous immunoassay using the immunoactivation method for the rapid and specific determination of human granulocyte elastase (EC 3.4.21.37) in plasma. The method uses anti-elastase antibody fragments from sheep, conjugated to horseradish peroxidase. These enzyme-antibody conjugates bind to the elastase-α_1-proteinase inhibitor complex present in plasma. A separate sample blank with non-specific sheep antibody fragments conjugated to horseradish peroxidase corrects for errors introduced by the sample matrix. Measurements were performed with the clinical chemistry analyser Hitachi® 717. A single determination can be performed in 10 min, requiring 24 µl sample volume. The measuring range is about 20 to 1000 µg/l elastase. For within-run precision the coefficients of variation are 4.77%, 4.48% and 1.85% for elastase concentrations of 45.7, 89.1 and 385.4 µg/l; for day-to-day precision the coefficients of variation are 15.81%, 7.19% and 4.12% for elastase concentrations of 31.1, 65.5 and 440.2 µg/l, respectively. Correlation ($y = bx + a$) of results with those from the heterogeneous immunoassay showed a good agreement ($r = 0.93$, $b = 1.11$, $a = -27.0$, $N = 121$). Interferences by endogeneous substances and by drugs at therapeutic doses were not observed. The reference interval, determined by using plasma from 215 healthy individuals (C-reactive protein < 5 mg/l, leukocyte count $4-8 \times 10^9$/l), was 9–56 µg/l (2.5th to 97.5th percentile), with a median of 27 µg/l.

Introduction

Severe injuries or infections are accompanied by a number of inflammatory reactions, the so-called inflammatory response. Among these reactions are the activation of humoral systems and the stimulation of inflammatory cells such as the polymorphonuclear granulocytes, which are well-equipped with proteolytic enzymes, like elastase[1]) (1–3). Thus, the increase in elastase in the plasma at an early stage reflects the intensity of the inflammatory stimulus (4). As a result of the rapid but graduated reaction of the granulocytes, the lysosomal elastase represents a key parameter in the diagnosis of traumatic and inflammatory processes (5–12). Elastase is not detectable in the plasma in its free state, but is complexed by potent inhibitors. About 90% of all circulating elastase is present as a stable 1:1 complex with α_1-proteinase inhibitor (13, 14). This complex can be determined in plasma samples using a homogeneous enzyme immunoassay. The test principle of the homogeneous assay, the immunoactivation method, exploits the fact that, in the presence of high concentrations of hydrogen peroxide, the enzymatic activity of polymeric forms of horseradish peroxidase, after binding to the elastase/α_1-proteinase inhibitor complex, is greater than that of the monomeric forms (15–18). Any interference by the sample matrix is compensated by a separate sample blank. This homogeneous assay therefore makes possible the determination of elastase on clinical chemistry analysers. The evaluation of the test on the Hitachi® 717 selective analyser is reported.

[1]) Enzyme: Polymorphonuclear granulocyte elastase, EC 3.4.21.37

Materials and Methods

Elastase was determined with the PMN Elastase IMAC (immunoactivation) immunoassay (E. Merck, Darmstadt, Germany). The kit contains antibody conjugate, blank conjugate, substrate B (1.5 mmol/l 4-aminophenazone, 0.1 mol/l phosphate buffer, pH = 7.0), substrate A (80 mmol/l hydrogen peroxide, 50 mmol/l phenol), and diluent for substrate A (20 mmol/l TRIS buffer, pH = 7.5). The antibody conjugate consists of sheep antibody fragments (Fab') against human elastase covalently bound to horseradish peroxidase, whereas the blank conjugate consists of non-specific sheep antibody fragments (Fab') covalently bound to horseradish peroxidase. For use on the Hitachi® 717 selective analyser (Boehringer Mannheim, Mannheim, Germany) one bottle each of lyophilized antibody conjugate and of blank conjugate are dissolved in 10 ml substrate B, forming the first reagent to be pipetted (Reagent 1). Substrate A is diluted 1:2 with the diluent for substrate A, forming the start reagent (Reagent 2). Table 1 shows the essential parameters for the determination on the Hitachi® 717. For the purpose of test interpretation, 2 channels are used, i.e. one for the sample and one for the sample blank. After calibration of the sample channel with the commercially available calibrator (E. Merck, Darmstadt, Germany), the resulting calibration factor is read into the sample blank channel ("Calibration List" in the "Monitor Job" menu). The Hitachi® 717 makes it possible to calculate the interference-corrected concentration of elastase in the sample by automatically calculating the difference between the two channels.

Tab. 1. Hitachi® 717 parameter setting.

Chemistry parameters	
Test	Elastase
Assay code	Rate
Sample volume, µl	12
Reagent 1 volume, µl	90
First incubation, s (points)	300 (1–25)
Reagent 2 volume, µl	180
Second incubation, s (points)	120 (26–34)
Measuring window, s (points)	72 (35–41)
Wavelength, nm	505
Calibration method	Linear
Standard (1), µg/l	0
Standard (2), µg/l	398
Reaction course	Increase

The sample channel was calibrated by a linear 2-point calibration, the resulting factor was inserted into the blank channel (modifications: Calibration Method = Factor).

A sandwich ELISA for the determination of elastase (E. Merck, Darmstadt, Germany) was used for method comparison. The calibrators of this heterogeneous assay, formerly containing only about ⅓ of the given elastase content, are now based on and recalibrated to (from batch #92019 onward) the same highly purified standard material also used in the PMN Elastase IMAC immunoassay.

As sample material, sodium citrate plasma (38 g sodium citrate per litre in the ratio 1 part citrate to 9 parts blood) was obtained by venipuncture. The plasma was separated by centrifuging at 1700 *g* for 10 min at room temperature. Samples which were not measured immediately were stored in plastic containers at −70 °C. All dilution of samples was performed with the IMAC sample dilution medium (E. Merck, Darmstadt, Germany).

C-reactive protein was determined in sodium citrate plasma on the Behring nephelometer analyser (Behring, Marburg, Germany). The reagent used was the NA Latex CRP Reagent (Behring, Marburg, Germany). In this assay polystyrene particles coated with antibodies to C-reactive protein are agglutinated by C-reactive protein in the patients' plasma. The scattered light signal obtained on the Behring nephelometer increases in relation to the concentration of C-reactive protein (measurement range: 2.5–160 mg/l, reference range for adults: < 10 mg/l). The white blood cells were counted using K-EDTA whole blood on the S-plus counter (Coulter, Luton, UK; reference range: 4–10 × 10^9/l).

Results

The test of linearity by serial dilution of a pooled plasma showed a dependence on the concentration of the calibrator. The lines in figure 1 show that after calibration with high calibrator concentrations the calculated regression lines (19) deviate only slightly from the origin.

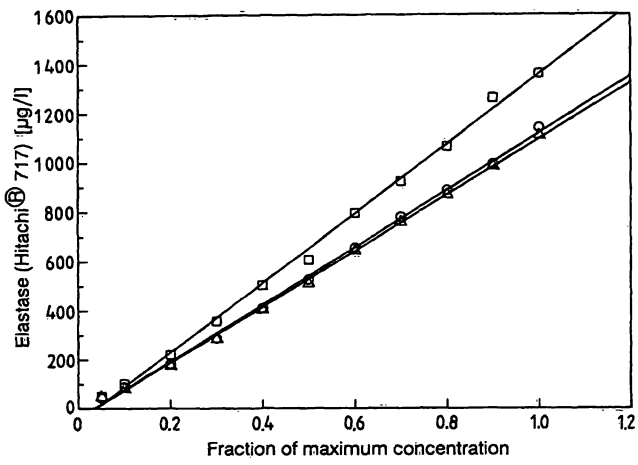

Fig. 1. Influence of the calibrator concentration on the linearity of the elastase assay. Serial dilutions of a highly concentrated plasma sample were determined in three runs using one calibrator concentration in each (□ 100 µg/l, ○ 398 µg/l and △ 960 µg/l). A regression analysis yielded slopes of 1410 (100 µg/l), 1158 (398 µg/l), 1134 (960 µg/l), and intercepts of −52.7 (100 g/l), −38.8 (398 µg/l), −38.5 (960 µg/l).

The imprecision of the method was studied by determining the within-run and day-to-day precision. The results obtained with various plasma pools are shown in table 2. On the basis of the convincing precision data the test can be performed as a single determination.

Tab. 2. Precision of the elastase determination performed with the Hitachi® 717 analyser.

	Elastase concentration [µg/l]		Imprecision	Number of samples
	mean	SD	CV [%]	
Within-run	45.7	2.2	4.77	20
	89.1	3.1	3.48	20
	385.4	7.1	1.85	20
Day-to-day	31.1	4.9	15.81	22
	65.6	4.7	7.19	22
	440.2	17.9	4.12	22

Day-to-day precision was performed with a single calibration, reflecting a calibration stability over 6 weeks.

The method was tested by comparing the immunoactivation test on the Hitachi® analyser with the commercial heterogeneous enzyme-linked immunosorbent assay (fig. 2). The evaluation of the results according to a distribution-free regression analysis (19) showed a good correlation ($y = bx + a$; $b = 1.11$, $a = -27.9$, $r = 0.93$, $N = 121$). Older batches of the heterogeneous assay yielded concentrations 3 times higher than those given by the IMAC assay. After conversion of the ELISA calibrators for the highly purified raw materials also used in the immunoactivation assay, and recalibration of the heterogeneous assay, similar results were obtained with both tests.

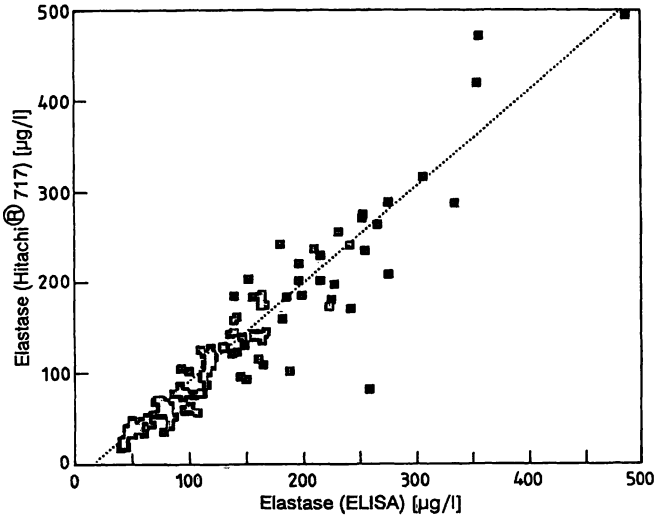

Fig. 2. Comparison of the homogeneous elastase assay on Hitachi® 717 analyser vs. a commercial ELISA according to a non-parametric regression analysis (19) ($y = bx + a$; $b = 1.11$, $a = -27.0$, $r = 0.93$, $N = 121$).

Analysis of lipaemic and haemolytic samples revealed that lipaemic samples with triacylglycerol concentrations up to 5 g/l and haemolytic samples with a free haemoglobin concentration up to 1 g/l in the plasma do not influence result. The same holds for hyperbilirubinaemia (plasma concentrations up to 510 µmol/l bilirubin). However, it must be noted that there is a lag phase in the time course of absorbance. This is the reason why we have chosen measurement points 35–41 when adjusting the parameters (fig. 3). Ascorbic acid in concentrations appreciably higher than physiological levels (after administration of multivitamin infusions) also causes noticeable interference. A possible explanation for this is that primary oxidation products of the substrate are rapidly reduced by the ascorbic acid (17).

Because of the high concentration of hydrogen peroxide used, the instrument was investigated for any carry-over effects via the reagent pipettes and cuvettes. No influence on other test methods (enzyme and substrate determinations), observable as a deviation from the within-run precision achieved by the instrument or the method-dependent blank value,

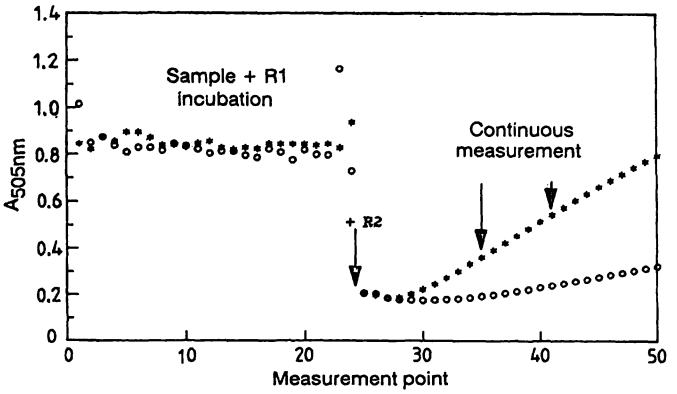

Fig. 3. Reaction monitor of the Hitachi® 717 analyser with a plasma sample containing 542 µmol/l bilirubin (* sample channel, o blank channel). Cuvette position = measurement taken every 12 seconds; R1 = addition of antibody-conjugate, respectively blank-conjugate; R2 = addition of substrate A.

could be detected. Citrate plasma samples for the determination of elastase can be frozen for months without any loss, or stored for 24 h at 4–8 °C (20). Samples, which cannot be centrifugated immediately, may be stored at room temperature for up to 5 h after venipuncture; during this storage period, falsely high values that might arise from a release reaction of the granulocytes are not observed. Because of the separate storage of the substrates (4-aminophenazone in Reagent 1, hydrogen peroxide and phenol in Reagent 2), the reagent used remains stable for weeks when cooled in the instrument (4–8 °C). The stability of the reagent was studied over six weeks by recording day-to-day precision for this period. The results are summarized in table 2.

To establish a reference range, citrate plasma samples were measured from 215 healthy adults (93 women, 122 men) aged 18 to 60 years with a C-reactive protein concentration < 10 mg/l and a leukocyte count of $4-10 \times 10^9$/l (fig. 4). The median was 27 µg/l and the reference range was 9 to 56 µg/l (2.5^{th} to 97.5^{th} percentile).

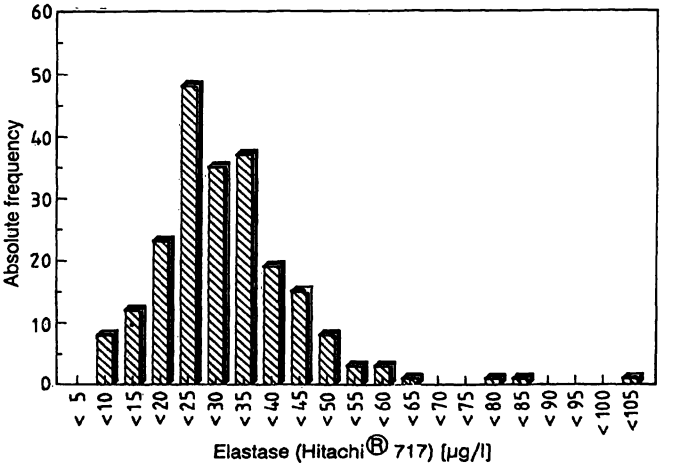

Fig. 4. Normal values of elastase determined from 215 healthy subjects (median: 27 µg/l, 2.5^{th}–97.5^{th} percentile: 9–56 µg/l).

Discussion

The availability of suitable markers for the early diagnosis of complications in the course of inflammatory processes facilitates more rapid and rational therapeutic treatment. Due to the fact that these parameters are in the µg/l range or lower, heterogeneous assays have been the methods of choice. However, these techniques are rather time-consuming and not really suitable for an emergency. The new immunoactivation technique offers the advantage of a determination of elastase in the plasma in less than 15 min. The test principle is based on the known fact that hydrogen peroxide converts monomeric forms of horseradish peroxidase via the active intermediate stages compound I and compound II into the enzymatically less active compound III. The oxidation states and coordination geometrics of the different species have been elucidated by spectrophotometric and X-ray diffraction experiments (21). The native enzyme is regenerated by the reaction of compound III with electron donors (e.g. phenol) via the intermediate stages compound I and compound II. Depending on the nature of the substrate, high H_2O_2 concentrations can lead to an accumulation of compound III and thus to a strongly decreased enzymatic activity of monomeric horseradish peroxidase. In the case of polymerized or aggregated horseradish peroxidase, a much lower accumulation of compound III is observed under the same conditions, so that the enzymatic activity is appreciably higher (17, 18).

This phenomenon, which is not yet clear in detail, forms the basis of the immunoactivation method. By linking anti-elastase antibody fragments to horseradish peroxidase, monomeric conjugates are obtained, which have a low enzymatic activity in the presence of a mixture of the substrates phenol and 4-aminophenazone, as well as a high concentration of hydrogen peroxide. If the monomeric conjugates first react with elastase from the sample, high-molecular immunoaggregates appear, with appreciably higher enzyme activity. The elastase concentration in the sample is directly proportional to the increase in enzymatic activity. All horseradish peroxidase effectors and substances that exert intrinsic peroxidase activity are potentially capable of interfering. These disturbances are compensated by the use of a special blank conjugate, so that influence of the sample matrix can be corrected (15, 16).

To perform the homogeneous assay on clinical chemistry analysers, it is necessary to occupy two channels (sample and blank). There is a linear relationship between the elastase concentration and the enzymatic activity of the antibody conjugate after complexing: this is determined by a 2-point calibration (standard 1: 0 µg/l, standard 2: 398 µg/l). In accordance with the nature of the blank conjugate, which is incapable of forming immunoaggregates and thus shows no dependence on the measurement signal from the standard concentration, the blank channel cannot be calibrated in a similar way. To make it possible to base both channels on an identical linear calibration, the factor for the sample channel obtained from the instrument is therefore used for the blank channel. The reagent blank value of the blank conjugate is then determined. Using this type of calibration ensures that both channels can assign the same measurement signals to the same concentration values. Interfering influences of the sample matrix, which cause identical modification of the measurement signal in both channels, can now be eliminated by simply subtracting the concentration values of one channel from those of the other.

Free elastase does not occur in the plasma. It immediately forms complexes with inhibitor, which is present in large excess in the plasma but not in the tissues. In addition to the elastase complexed with the α_1-proteinase inhibitor in the plasma, there is a small fraction consisting of a complex with the α_2-macroglobulin (total fraction $< 10\%$) (13, 14). This complex does not bind with the antibody conjugate of the homogeneous test (*Dreher, M.*, unpublished data). For this reason, for plasma, the specificity of the test does not differ from that of the heterogeneous ELISA, which has been used up to now.

The latter is a typical sandwich immunoassay utilizing antibodies directed against elastase and α_1-proteinase inhibitor (22). A comparison of both methods shows a good correlation. The within-run precision and day-to-day precision show coefficients of variation that are typical of tests performed on analysers. The linearity over the extended measurement range (up to 1000 µg/l) requires additional dilution only in rare cases of massive elastase release. Apart from a few extreme cases (e.g. high bilirubin, high ascorbic acid), no false results have been found. No carry-over effects of the reagent on the analyser could be detected. On the basis of the long stability of the reagent and the satisfactory stability of the sample in vitro without special sample collection and work-up, the determination method is suitable for emergency testing.

Conclusion

The determination of elastase/α_1-proteinase inhibitor complex in the plasma is a sensitive, precise routine assay, linear over a wide range of measurement, which

can be performed in less than 15 min on the Hitachi® 717 automated analyser without special treatment of the sample.

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